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A Chimeric IgG4 Monoclonal Antibody Directed Against CD18 Reduces Infarct Size in a Primate Model of Myocardial Ischemia and Reperfusion

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Objectives. **This study attempted to determine whether neutrophil sequestration in reperfused myocardium can be inhibited and infarct size reduced by treatment with a chimeric, monoclonal IgG4 antibody (CLB54) directed against CD18 in a primate model of acute myocardial ischemia and reperfusion.**

Background. **Reperfusion injury, in part mediated by neutrophils, may limit the potential benefit of reestablishing infarct-related artery patency in patients with acute myocardial infarction.**

Methods. **Nineteen closed-chest baboons (10 control, 9 treated with CLB54) had the left anterior descending coronary artery occluded for 90 min, followed by 4 h of reflow. CLB54 (mean [±SD] 11 ± 2 mg/kg body weight) or saline solution was administered intravenously 20 min before reflow. Coronary flow was determined using radiolabeled microspheres, infarct size by triphenyltetrazolium chloride staining, global and regional ventricular function by contrast ventriculography and neutrophil accumulation by a myeloperoxidase assay.**

Results. **Risk region size was the same in both groups. CLB54 treatment reduced infarct size expressed as a percent of the risk region from 41 ± 20% in the saline-treated group to 19 ± 17% in the CLB54-treated group ($p < 0.02$). This was associated with diminished myeloperoxidase activity and greater postreperfusion coronary flow in the risk region in CLB54-treated than in control baboons. Ejection fraction declined to the same extent in both groups, whereas anterior wall regional cord shortening was better preserved in CLB54-treated baboons.**

Conclusions. **Inhibition of neutrophil sequestration with CLB54 administered before reperfusion reduces infarct size, preserves ischemic zone microvascular perfusion and minimizes the decline of regional wall motion.**

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In acute myocardial infarction, establishing patency of the occluded coronary artery with thrombolytic therapy has beneficial effects on left ventricular function and mortality (1–3). Nevertheless, studies in cardiac and noncardiac animal models of ischemia and reperfusion (4–9) suggest that vascular and parenchymal tissue injury can occur as a result of reperfusion itself, thus reducing the potential benefit of restoring flow. For this reason, strategies designed to minimize reperfusion injury have been sought.

A number of factors and cell types are thought to contribute to reperfusion injury (10–13). Although these mechanisms are part of the normal process of "inflammation and repair," in reperfusion injury these pathophysiologic processes are considered to have "gone awry," extending necrosis more than promoting repair. The polymorphonuclear leukocyte has been the focus of particularly intense study. Potentially deleterious effects of neutrophils in reperfused tissue include direct parenchymal tissue injury through generation of free radicals (10) and release of lysosomal enzymes and injury secondary to effects on the microcirculation (11–16).

Neutrophil adhesion to endothelial cells is mediated by a number of specific adhesion molecules, including L-selectins and the CD11/CD18 family of beta-integrins and their endothelial cell counterreceptors (17). Because the beta-integrin family of adhesion molecules appears to mediate firm attachment of neutrophils to endothelial cells through the endothelial cell counterreceptor intercellular adhesion molecule (ICAM)-1, and because such attachment is a necessary first step to subsequent diapedesis, monoclonal antibodies directed against CD11/CD18 have therapeutic potential in reducing the neutrophil-mediated component of reperfusion injury. The present study was undertaken to test the anti-CD18 antibody CLB54 as a therapy to reduce infarct size in a primate model of acute ischemia followed by reperfusion.

In preliminary studies, the appropriate dose of CLB54 was determined by a combination of in vitro and in vivo dose–response testing. After determining the appropriate dose of CLB54, an infarct size study was performed that showed that administration of CLB54 just before reflow reduces infarct size by 50% by a mechanism associated with reduced neutrophil sequestration in previously ischemic reperfused myocardium.

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CLB54 treatment was also associated with preservation of microvascular perfusion and regional myocardial function in the ischemic zone.

Methods

The chimeric CLB54 monoclonal antibody used in this study is a human/mouse genetic reconstruction of a murine monoclonal IgG4 molecule that binds selectively to the neutrophil CD18 receptor. It was supplied as a sterile, nonpyrogenic solution of 5 mg of monoclonal IgG4 per milliliter of buffer solution containing 0.15 mol/liter of sodium chloride, 0.01 mol/liter of sodium phosphate and 0.01% of polysorbate 80 at pH 6.5 (Centocor, Inc.).

In vitro methods. *Neutrophil isolation.* Five milliliters of heparinized blood was diluted 10-fold with ice-cold buffer (0.155 mol/liter ammonium chloride, 10 mmol/liter potassium bicarbonate, 0.1 mmol/liter ethylenediaminetetraacetic acid [EDTA], pH 7.4) and incubated 10 min on ice to lyse red cells. Cells were pelleted 5 min at 1,500 rpm, resuspended in 10 ml of cold lysis buffer, centrifuged and resuspended in 0.5 ml of Hank's balanced salt solution (HBSS)/5 ml of starting blood volume.

Neutrophil adhesion assay. Human umbilical vein endothelial cells (HUVECs) (Cell Systems) were obtained at passage 1 and cultured and used at passage 4. Cells were grown to confluence on gelatin-coated 96-well plates and stimulated with 50 μg/ml of tissue necrosis factor-alpha (Genzyme) for 24 h.

Medium was removed from the HUVECs and replaced with HBSS containing 5 mg/ml of human serum albumin; 50 μl of a 4.0×10^6 cells/ml suspension of neutrophils was added to the wells for 15 min at 37°C. Unbound cells were removed by two 200-μl HBSS washes. Cells were solubilized with 200 μl of 0.5% hexadecyltrimethylammonium bromide (HTAB) in 50 mmol/liter potassium phosphate at pH 6.0 (HTAB buffer). Bound neutrophils were quantified by myeloperoxidase assay.

Myeloperoxidase assay for neutrophil adhesion assay. The following solutions were sequentially pipetted into 96-well flat-bottomed polystyrene microtitration plates (volumes are per well): 15-μl sample; 55 μl of 80 mmol/liter potassium phosphate buffer, pH 5.4; 20 μl of 0.3 mmol/liter hydrogen peroxide in 80 mmol/liter potassium phosphate buffer, pH 5.4; 10 μl of 16 mmol/liter 3,3′,5,5′-tetramethylbenzidine (Sigma Corp.) *N,N*-dimethylformamide. Plates were developed for 10 min at room temperature and then stopped with 100 μl/well of 1 mol/liter phosphoric acid and optical density read at 450 nm.

Iodine-125–labeled CLB54 binding to neutrophils. Neutrophils were resuspended in HBSS to 4.0×10^6 cells/ml and aliquoted 50 μl/well into 96-well polyvinyl chloride plates. Neutrophils were incubated for 60 min at 37°C with iodine-125–labeled CLB54 (specific activity 2 μCi/μg) added to a final concentration of 40 μg/ml. Cells were washed with 200 μl of HBSS and pelleted three times. The cell pellet was finally solubilized and counted in a gamma counter.

Quantification of free antibody levels. Ninety-six-well enzyme-linked immunosorbent assay (ELISA) plates were coated overnight with 10 μg/ml of rabbit anti-CLB54 IgG in coating buffer (1.6 g/liter of anhydrous sodium carbonate, 2.93 g/liter of sodium bicarbonate) and then washed three times with 250 μl/well of ELISA wash buffer (9 g/liter of sodium chloride, 0.2 g/liter of Tween-20). Plates were blocked 1 h at 37°C with 200-μl/well human anti-mouse antibody (HAMA) diluent (45% goat serum, 35% fetal bovine serum, 20% phosphate-buffered serum). Samples were added 50 μl/well, initially diluted 1:10 in HAMA diluent and further diluted 1:2 in HAMA diluent with the final dilution of 1:640, for 1 h at room temperature. A standard curve of chimeric antibody (50 to 0.006 μg/ml) in baboon plasma was also tested. Plates were washed three times with 250 μl of ELISA wash buffer. Then, 50 μl of alkaline phosphatase conjugated antihuman H+L antibody (1 μg/ml) was added to each well and incubated for 1 h at room temperature. After three washes with 250 μl of ELISA wash buffer, 100 μl of alkaline phosphatase substrate was added to each well and allowed to incubate 15 min at room temperature. The reaction was stopped with 50 μl of 3 *N* sodium hydroxide, and the optical density at 405 nm was read. The optical densities of samples that fell on the linear portion of the standard curve were used to calculate the concentration of free antibody in the plasma.

In vivo methods. *Reverse Arthus reaction.* Ten baboons were tranquilized with ketamine (15 mg/kg body weight intramuscularly), and an extremity vein was cannulated with a standard 21-gauge butterfly needle; 15 mg/kg of bovine serum albumin (Sigma Corp.) was administered intravenously. The skin on one or both forearms was shaved, 0.1 to 0.2 ml of saline solution or rabbit anti–bovine serum albumin (Sigma Corp.), minimum 2 mg of anti–bovine serum albumin/ml) was injected intradermally using a 25-gauge tuberculin syringe, and the injection sites were marked and labeled. Sites were examined grossly, and full-thickness skin biopsies were performed at 24 h. Six control baboons received no CLB54; one baboon was treated with 1 mg/kg, one with 3 mg/kg, one with 9 mg/kg and one with 11 mg/kg of CLB54 administered intravenously just after bovine serum albumin administration. Blood samples for CLB54 concentration were obtained before and 5 min and 1, 3, 5 and 24 h after CLB54 administration. Development of edema, induration and hemorrhage at the site of anti–bovine serum albumin injection was monitored over 24 h, and the reaction was graded semiquantitatively on a 0 (no reaction) to 4+ (edema, hemorrhage, induration) scale. At 24 h full-thickness biopsy samples of the lesions were obtained and were stained with hematoxylin–eosin. Neutrophil infiltration was graded on a 0 (normal biopsy) to 4+ (sheets of neutrophils, hemorrhage and edema) semiquantitative scale.

Infarct model. This infarct model was used in both a preliminary dose-finding study and in the final infarct-size study (see Results).

Baboons (20 to 30 kg) of either gender were tranquilized with ketamine (15 mg/kg) and were intubated and ventilated with supplemental oxygen and 1% to 2% halothane inhalation

anesthetic. Halothane concentration was adjusted to keep the baboons anesthetized throughout the experiment while maintaining stable arterial blood pressure.

Both femoral arteries and the right femoral vein were isolated by direct exposure and cannulated with 7F sheaths (Cordis, Inc.). The arterial sheaths were used to introduce various required catheters, and their sidearms were used for reference sample withdrawal for microsphere measurements of coronary blood flow (see later). The venous sheath was used for administration of fluid and drugs as required. After femoral vessel cannulation, 10,000 IU of heparin was administered intravenously. For coronary angiography and placement of the angioplasty balloon (see later), a 7F angioplasty guiding catheter (standard AL-I or custom-made JL-2.5 [Cordis, Inc.]) was advanced over a 0.035-in. (0.89 mm) guide wire into the ascending aorta through a femoral artery sheath and the left coronary ostium engaged. For left ventriculography, aortic pressure measurement and microsphere administration, a 6F pigtail catheter (Cordis, Inc.) was advanced over a 0.035-in. guide wire into the ascending aorta through the other femoral artery sheath. The pigtail catheter could be advanced retrogradely into the left ventricle or withdrawn into the ascending aorta, as needed.

End-expiratory carbon dioxide, lead I or II of the electrocardiogram (ECG) and aortic pressure were monitored throughout the procedure.

Left ventriculography. Left ventriculography was performed in the 45° right anterior oblique projection. The pigtail catheter was advanced into the left ventricular cavity, and 6 to 8 ml/s of nonionic contrast was injected for a total of 24 to 32 ml through the pigtail catheter using an automatic, calibrated power injector (MedRad, Inc.). The ventriculogram was recorded cineangiographically at 30 frames/s, and a calibration grid was filmed at heart level immediately afterward.

For analysis, a single end-diastolic and end-systolic frame of the baseline and end-study contrast ventriculogram was digitized, and the ventricular contours were traced using a mouse cursor and a commercial digitizing package (Image Comm, Inc.). The end-diastolic and end-systolic left ventricular volumes were determined using the filmed grid for calibration (single-plane modification of the Sandler–Dodge area–length method [18]), and stroke volume and ejection fraction (stroke volume expressed as a percent of end-diastolic volume) were calculated. Regional chord shortening along 100 chords from the base of the anterior wall to the base of the inferior wall was determined, as previously described (19). The right anterior oblique left ventricular contour was divided into five segments (anterobasal, midanterior, apical, midinferior and inferobasal) of 20 chords each, and the median chord shortening was determined for each region. Regional function was defined as the median chord shortening for each of these regions.

Microsphere blood flow. For microsphere blood flow measurements, the pigtail catheter was placed in the left ventricle. Reference sample withdrawal from the sidearm of a femoral artery sheath was begun at 2.16 ml/min using a calibrated withdrawal pump (Harvard, Inc.) just before microsphere administration. Then, 1 to 2 million 15-μm microspheres labeled with Gd-152, Sn-113, Sc-46 or Nb-95 were administered into the left ventricle through the pigtail catheter. The catheter was then withdrawn into the aorta, and aortic pressure and the ECG were recorded. Reference sample withdrawal was terminated no sooner than 2 min after microsphere injection.

Regional coronary blood flow in myocardial samples (see later) was determined by counting each myocardial and reference sample for 10 min in a gamma counter and correcting counts for background and overlap. Coronary blood flow (CBF) in a myocardial sample was then given as follows: CBF (ml/min/g) = cpm/g (tissue)/cpm (reference) $\times$ 2.16 ml/min, where cpm/g (tissue) = corrected counts per minute per gram tissue, and cmp (reference) = corrected counts per minute in the reference sample.

Protocol. Aortic or left ventricular pressure, heart rate and the ECG were monitored continuously throughout the procedure and recorded periodically. The pigtail catheter was advanced into the left ventricle, and a right anterior oblique ventriculogram obtained. Cineangiography of the left coronary circulation was performed in the right and left anterior oblique projections, and the approximate size of the proximal left anterior descending coronary artery was estimated, using the diameter of the 7F angioplasty guiding catheter for calibration. The appropriate-sized angioplasty balloon (Slider ST, Mansfield, Inc.) was selected and was advanced using fluoroscopic visualization over a 0.014-in. (0.36 mm) guide wire into the proximal left anterior descending artery. The balloon was then inflated to 3 atm of pressure (45 psi). Coronary occlusion was confirmed with contrast cineangiography initially and periodically throughout the protocol.

Twenty minutes before occlusion release, either saline solution (control baboons) or CLB54 (treated baboons) was administered intravenously as a bolus.

Before occlusion release, a final cineangiogram was obtained, microsphere coronary flow was measured and aortic and left ventricular pressure and the ECG were recorded. The angioplasty balloon was then deflated and removed with the guide wire from the coronary artery. Serious ventricular arrhythmias (sustained ventricular tachycardia or ventricular fibrillation requiring defibrillation) were treated with a single dose of lidocaine, 30 mg intravenously. This was repeated only if serious arrhythmias returned. No baboon was treated with continuous infusion of lidocaine.

Ten minutes and 4 h after occlusion release, cineangiograms, microsphere coronary flow, systemic pressures and the ECG were recorded. Just before sacrifice, the right anterior oblique left ventriculography was repeated. Animals were killed by intraventricular administration of hyperkalemic solution through the pigtail catheter.

Estimation of risk region and infarct size. After sacrifice, the heart was removed, the right ventricle and both atria were trimmed, and the heart was cut transversely into five to six 1-cm thick rings from base to apex, which were weighed and placed for 15 min in 1% triphenyltetrazolium chloride to

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distinguish infarcted from noninfarcted myocardium. The mass of infarcted and noninfarcted tissue was estimated by digital planimetry of color 35-mm slides of the stained myocardial rings. The ratio of planimetered infarct area to planimetered total area for each ring was multiplied by ring weight to determine infarct mass for each ring; infarct masses for all rings were then summed to determine total infarct mass. Infarct mass as a percent of the left ventricular mass (weighed) was calculated from these measurements.

Risk and nonrisk regions were defined by microsphere blood flow. Hearts were sectioned into 70 to 100 samples, and blood flow at the end of coronary occlusion for each sample was determined. A sample was defined as being from the area at risk if flow was <50% of flow in the nonrisk region. Risk region mass was calculated by summing the weights of all risk region samples so defined. Infarct mass as a percent of risk mass and risk region mass as a percent of the left ventricular mass were calculated, using infarct mass determined by digital planimetry and total left ventricular mass by direct weighing.

Regions of no or low reflow were defined as samples taken in the risk region that at 4 h of reflow had <50% of simultaneously determined nonischemic zone coronary flow.

Myeloperoxidase assay. Myeloperoxidase activity was measured as previously described (20). After triphenyltetrazolium chloride staining, 50- to 150-mg samples were obtained from the endocardium and epicardium of the central ischemic and the nonischemic regions and were weighed and frozen at −70°C. Samples were pulverized under liquid nitrogen, homogenized and sonicated and then underwent three rounds of freeze–thaw to release myeloperoxidase. Myeloperoxidase activity was measured by adding *O*-dianisidine hydrochloride and hydrogen peroxide and then observing the change in light absorbance at a wavelength of 460 nm for 2 min. Results were expressed as units of myeloperoxidase per 100 mg wet weight of the samples.

Statistics. All data are reported as mean value ± SD. Comparisons of two means were performed using an unpaired Student *t* test if the data were normal and a Mann–Whitney nonparametric test for significance for data that were not normal. When more than two means were compared, analysis of variance was performed first, and a *t* test corrected for multiple comparisons was performed to detect significant differences between any two mean values. All statistics were performed using Systat version 5.0 (Systat, Inc.).

Results

Preliminary studies. *In vitro antibody characterization.* There was a 100-fold difference between the concentration of CLB54 required for half-maximal occupancy of CD18 receptor sites on neutrophils and the concentration required in whole blood for half-maximal inhibition of neutrophil adhesion. The concentration of CLB54 required for half-maximal occupancy of CD18 receptors in isolated human neutrophils was 0.2 μg/ml. Half-maximal inhibition of isolated human neutrophil adhesion to tumor necrosis factor-alpha–stimulated HUVECs was 10 times higher at 2 μg/ml. Treatment of whole human blood with chimeric anti-CD18 required 20 μg/ml of antibody for half-maximal inhibition of neutrophil binding to tumor necrosis factor-alpha–stimulated HUVECs.

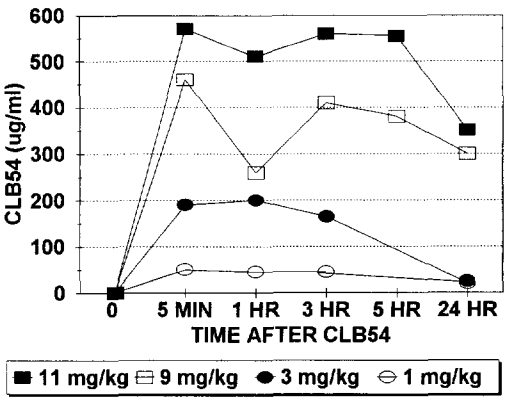

Figure 1. Serum blood levels after four different intravenous doses of CLB54 in four different baboons.

In vivo antibody characterization. In vivo characteristics of CLB54 were examined in several dose–response studies.

Plasma levels of free antibody achieved at various doses of CLB54 (from four baboons that received CLB54 in the reverse passive Arthus reaction) are shown in Figure 1. At the higher doses, CLB54 levels increased rapidly and were sustained for 24 h after administration. Neutrophil count increased significantly after administration of CLB54 at the higher dosage.

Reverse passive Arthus reaction. The reverse passive Arthus reaction was associated with moderate gross and histologic evidence of inflammation in non–CLB54-treated animals (2.3 ± 1.0 and 2.5 ± 1.5 reaction grade, respectively, on a scale of 0 to 4). Neither 1- nor 3-mg/kg doses of CLB54 reduced either gross or histologic evidence of inflammation. However, in both baboons treated with an average of 10 mg/kg of CLB54 (9 and 11 mg/kg), the reaction was completely abolished grossly (0 reaction grade in both) and minimized histologically (1 reaction grade in both).

Preliminary infarct studies. In preliminary studies, an average dose of 5 ± 4 mg/kg in six baboons failed to reduce infarct size, expressed as a percent of risk region, compared with results in the control animals (control group 41 ± 20% vs. CLB54 treatment 44 ± 22%) despite equal risk region sizes (~35% of the left ventricle). Early postreperfusion ischemic zone coronary blood flow expressed as a percent of nonischemic zone flow was the same in the control and CLB54-treated groups (113 ± 50% vs. 120 ± 21%). Neutrophil levels were measured in only three of six CLB54-treated baboons but were modestly reduced compared with control animals (1.6 ± 1.1 vs. 1.0 ± 0.3 U/100 mg left ventricle for control vs. CLB54-treated animals, respectively). On the basis of these preliminary data and the reverse passive Arthus reaction data, subsequent studies and analyses were performed using CLB54 doses ≥10 mg/kg. (Doses >10 mg/kg were used in two baboons to determine whether further infarct size reduction could be obtained at higher doses.)

Ex vivo antibody characterization. The effect of CLB54 (10 mg/kg, plasma concentrations of ~400 to 500 μg/ml) on binding of neutrophils to HUVECs from two baboons was studied ex vivo. Baboon blood samples were collected before and 5 (two animals) and 30 min (one animal) after antibody administration, and neutrophils were isolated. Neutrophil binding to both unstimulated and tumor necrosis factor-alpha–stimulated HUVECs was reduced by 50 ± 6% at 5 min and by 63% 30 min after CLB54 administration to levels comparable to control neutrophil binding to unstimulated HUVECs. Furthermore, binding of iodine-125–labeled anti-CD18 to the neutrophils isolated from whole-blood samples from CLB54-treated baboons revealed that there were no residual CD18 sites available for further antibody binding, suggesting 100% occupancy of CD18 by CLB54.

Infarct model. As a result of in vivo and ex vivo antibody characterization, subsequent studies and analysis were performed using CLB54 at doses ≥10 mg/kg. (Doses >10 mg/kg were used in two baboons to determine whether further infarct size reduction could be obtained at higher doses.)

A total of 22 baboons (11 control and 11 CLB54 treated) were studied. Two animals died at onset of coronary occlusion (one control, one CLB54 treated) and were not analyzed further. In one CLB54-treated baboon, the occluding balloon inflation pressure decreased during the occlusion period. Because it was unknown whether coronary flow occurred during the period of lowered pressure, data from this animal (which had an infarct smaller than the mean size for the CLB54-treated group) were not used. Ten control and nine CLB54-treated baboons completed the protocol. The CLB54-treated animals received an average dose of 11 ± 2 mg/kg (range 10 to 15).

Hemodynamic variables. Before coronary occlusion in control and CLB54-treated baboons, aortic systolic blood pressure was 101 ± 18 and 103 ± 15 mm Hg, aortic diastolic pressure 69 ± 10 and 73 ± 13 mm Hg, and heart rate 99 ± 12 and 109 ± 15 beats/min, respectively. Aortic blood pressures and heart rate did not change significantly during the course of the protocol and were not different in control and CLB54-treated baboons at any time.

Infarct size. Figure 2 summarizes risk region and infarct sizes in control and CLB54-treated baboons. Risk regions were not significantly different in the two groups (37 ± 9% vs. 41 ± 11%, control vs. CLB54-treated animals, respectively). CLB54 administered 20 min before reflow reduced infarct size by 50% from 41 ± 20% to 19 ± 17% of the area at risk ($p < 0.02$).

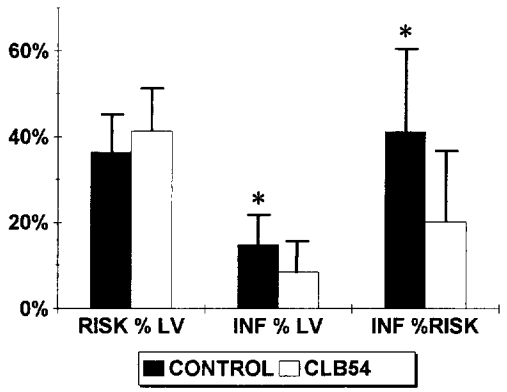

Figure 2. Risk region (RISK) and infarct (INF) size as a percent of the left ventricle (LV) and as a percent of the risk region in control and CLB54-treated baboons. *$p < 0.05$ compared with CLB54-treated animals.

Coronary blood flow. Coronary flow data are summarized in Table 1. Ten minutes and 4 h after reflow, coronary flow in the risk region was greater in CLB54-treated than control animals.

There was no relation between transmural coronary flow (collateral flow) in the risk region during occlusion and infarct size in either group.

The size of the no or low reflow region expressed as a percent of the ischemic zone was 36 ± 28% in control and 21 ± 25% in CLB54-treated baboons ($p = 0.10$).

Myeloperoxidase. Myeloperoxidase activity is summarized in Figure 3. Neutrophil accumulation was low in the nonischemic zone endocardial and epicardial layers of both control and CLB54-treated animals. However, in the risk region transmural myeloperoxidase activity expressed as a percent of nonischemic zone myeloperoxidase activity was 640 ± 420% in control baboons (or 1.6 ± 1.1 U/100 mg of the left ventricle) and 210 ± 60% (or 0.5 ± 0.2 U/100 mg of the left ventricle) in CLB54-treated animals ($p < 0.02$). The reduction of endocardial myeloperoxidase activity was of borderline significance ($p = 0.06$), but that of epicardial myeloperoxidase activity was significant ($p < 0.01$).

Reperfusion arrhythmias. Development and severity of reperfusion arrhythmias was not different in the two groups.

Table 1. Coronary Blood Flow in Ischemic and Nonischemic Regions

	Nonischemic Zone		Ischemic Zone	
	Control Group (ml/min/100 g LV)	CLB54 Group (ml/min/100 g LV)	Control Group (ml/min/100 g LV)	CLB54 Group (ml/min/100 g LV)
Occlusion	92 ± 28	90 ± 39	10 ± 6	8 ± 6
Early reflow*	154 ± 55	145 ± 74	197 ± 160	360 ± 209†
Late reflow*	76 ± 19	98 ± 31	48 ± 20	84 ± 36‡

*Test for significance was unpaired *t* test for late reflow data (normal data) and Mann–Whitney test for early reflow data (nonnormal data). See text for details. †$p < 0.06$ compared with control group (Mann–Whitney test). ‡$p < 0.03$ compared with control group (unpaired *t* test). Data presented are mean value ± SD. LV = left ventricle.

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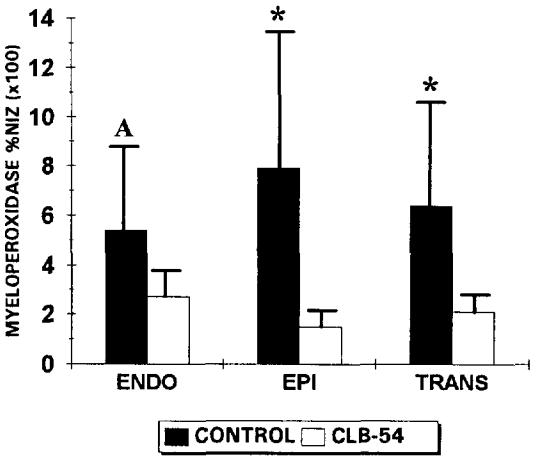


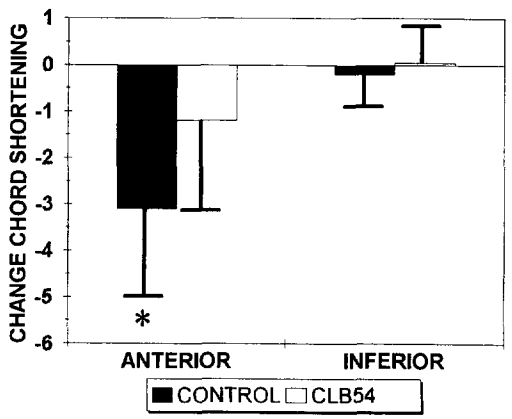
Figure 3. Myeloperoxidase activity in the ischemic zone expressed as a percent of myeloperoxidase activity in the nonischemic zone (NIZ). Myeloperoxidase values for endocardial (ENDO) and epicardial (EPI) regions and transmurally (TRANS) are shown. *p < 0.05 compared with CLB54-treated baboons. See text for details.

Four baboons developed ventricular tachycardia, and two had accelerated idioventricular rhythm in the CLB54-treated group; five animals developed ventricular tachycardia, and one had ventricular fibrillation in the control group. Four animals in each group received one 30-mg bolus of lidocaine at the onset of reflow for their arrhythmia. One animal in the CLB54-treated group received 1 mg of atropine intravenously for bradycardia at onset of reflow.

Ventricular function. Preocclusion ejection fraction was the same in both groups (51 ± 12% and 46 ± 11%, control and CLB54, respectively). Myocardial infarction was associated with a significant and identical decline (~17% absolute change in ejection fraction) in ejection fraction in the control and CLB54-treated baboons. Similarly, when regional function was assessed by median cord shortening in the five segments of the right anterior oblique ventriculogram, there was no difference between the two groups.

However, when anterior wall function was defined as the average cord shortening of the midanterior and apical segments, there was a significant difference between the groups in the decline in anterior wall function, the extent of decline in the CLB54-treated group being half that of controls (Fig. 4). However, there was no relation between infarct size, expressed as a percent of the left ventricle or risk region, and the decline in regional function. Function in the nonischemic inferior left ventricular wall was not different in the two groups.

Figure 4. Change in chord shortening in control and CLB54-treated baboons. Anterior chord shortening is the average of the midanterior and anteroapical chord shortening; inferior chord shortening is the average of the inferobasal and midinferior wall chord shortening. See text for details. *p < 0.05 compared with CLB54-treated animals.



Discussion

The present study shows that CLB54 administered within 20 min of reflow reduces infarct size and preserves regional function in short-term closed-chest primate model of myocardial ischemia and reperfusion. Reduction in infarct size was accompanied by a reduction in myeloperoxidase activity in the ischemic region, consistent with a mechanism involving inhibition of neutrophil sequestration. In addition, improved immediate and late postreflow coronary blood flow suggests preservation of ischemic region microvascular integrity with CLB54 treatment.

Relation to previous studies. A number of studies investigating the ability of anti–leukocyte-adhesion therapies have been performed in ischemia–reperfusion models, such as the rabbit ear (8), lung transplantation (5), hepatic ischemia and reperfusion (4), and myocardial stunning (6) and infarction (7,9). These studies demonstrated that tissue dysfunction or necrosis can be minimized by inhibition of neutrophil–endothelial cell adhesion using one of a number of anti-CD18 or anti-CD11 (a, b or c) monoclonal antibodies.

Several studies have used monoclonal antibodies directed against the CD11/CD18 heterodimer in an attempt to limit myocardial reperfusion injury. One preliminary study (21) in primates reported that R15.7, an anti-CD18 antibody, administered 30 min before coronary occlusion, reduced infarct size from 27% to 9% of the area at risk, improved regional function in the ischemic area as measured by sonomicrometry and reduced the incidence of ventricular fibrillation but did not improve the recovery of microvascular flow. That study (21) differs from ours in that anti-CD18 therapy was administered before vessel occlusion. In addition, we found an improvement in both early and late reperfusion coronary blood flow and no difference in the development of serious arrhythmias. Differences in timing of antibody administration and in the characteristics of the antibody, itself, may account for these disparate results. However, the studies are similar in their most important finding—that anti-CD18 therapy reduces infarct size in the primate.

Two studies have been reported in dogs. Simpson et al. (9) showed that an anti-Mo 1 (904, anti-CD11B) antibody reduced infarct size by 46% in dogs treated with 1 mg/kg. The reduction in infarct size was associated with a reduction in neutrophil accumulation in the risk region (9). These data are consistent

with data from the current study. The recent study of Tanaka et al. (22) showed a dissociation between neutrophil sequestration and improvement in microvascular flow on the one hand and infarct size on the other. In particular, despite a marked reduction in neutrophil sequestration and improved microvascular flow in anti-CD18–treated dogs, there was no reduction in infarct size (22).

The reason for the discrepancy in infarct size reduction between these two studies is unclear. Treatment with antiadhesion therapy inhibited neutrophil sequestration in both studies, yet infarct size was affected only in the study of Simpson et al. (9). This is particularly interesting because, as pointed out by Tanaka et al. (22), the antibody used in their study, 904, is directed against CD11b. Heterodimers consisting of CD18 associated with CD11a or CD11c would presumably not be inhibited. Why blocking only a single CD11 subtype would be effective while an anti-CD18 therapy, which should block all CD11/CD18 heterodimers known, would not (22) is unclear.

Other methods of interfering with the effect of neutrophils on reperfused myocardium have also been applied. Litt et al. (23) removed neutrophils mechanically, using filters just before reflow, and demonstrated significant myocardial preservation. More recently, Ma et al. (24) showed that inhibition of L-selectin–mediated neutrophil adhesion with a monoclonal antibody (DREG-200) directed against this adhesion molecule reduced infarct size in a feline model of myocardial ischemia and reperfusion. That study demonstrated that inhibition of the early, loose neutrophil adherence—a first step in neutrophil sequestration that appears to be necessary before firm CD11/CD18-mediated adherence can take place—can also reduce infarct size.

In the present study, anti-CD18 therapy with CLB54 reduced infarct size in the baboon independent of collateral flow. Previous studies (25) have noted a similar absence of a relation between collateral flow and infarct size in primates, most likely because primates have consistently low collateral flow (usually <10 ml/min per 100 g of left ventricle). In dogs, most studies have shown a close relation between collateral flow and infarct size (22,26), with the degree of collateral flow being more variable and greater than that seen in primates.

Interpretation. The hypothesis underlying the use of CLB54 for inhibition of neutrophil adhesion to endothelial cells is that reduction of neutrophil sequestration in the vascular space of an organ would prevent or minimize reperfusion-related parenchymal damage. The reduction in risk region neutrophil levels in CLB54-treated animals supports this hypothesis because neutrophil levels are proportional to tissue neutrophil density (20). The increase in early and late reflow in the risk region of CLB54-treated animals compared with controls provides evidence that inhibition of neutrophil adhesion by CLB54 also reduces the damage secondary to mechanical and biochemical effects of neutrophils on the microvasculature. Failure to reduce infarct size using more modest doses of CLB54 in preliminary studies was associated with an intermediate reduction in neutrophil levels and no preservation of microvascular perfusion.

Although these data support the primary hypothesis underlying the present study, it remains possible that the association among reduced neutrophil accumulation, improved microvascular flow and infarct size reduction is not directly causal. Perhaps in addition to reducing neutrophil sequestration, CLB54 treatment inhibits other destructive actions of those neutrophils that are sequestered. Despite CLB54 treatment, neutrophils still do accumulate in the risk region. Adequate blockade of CD18 with CLB54 may prevent these sequestered neutrophils from promoting further tissue damage perhaps by inhibiting diapedesis, neutrophil attachment to myocytes or perhaps by inhibiting steps leading to neutrophil release of lysosomal enzymes or generation of free radicals. This may also explain part of the discrepancy among studies of CD18 inhibition that uniformly show inhibition of neutrophil sequestration but differ in infarct size reduction.

The relation among CD18 occupancy, in vitro neutrophil adhesion inhibition and in vivo efficacy was evaluated in the present study. In vitro characterization of CLB54 showed half-maximal occupancy of CD18 binding sites at a level of 0.2 μg/ml. However, half-maximal inhibition of isolated neutrophil binding to HUVECs was achieved at a 10-fold higher dose (2 μg/ml); and half-maximal inhibition in whole blood required a concentration of 20 μg/ml. A level of ~50 μg/ml is achieved when 1 mg/kg is given to the baboon. Yet at more than twice the concentration required for half-maximal inhibition of neutrophil adhesion to HUVECs in whole blood, CLB54 failed to inhibit the reverse-phase Arthus reaction and failed to reduce infarct size in vivo in the baboon. Indeed, dosages <10 mg/kg (free antibody levels >400 μg/ml) of CLB54 failed to inhibit either of these effects. These data demonstrate that in vitro antibody characterization of both occupancy and neutrophil adhesion inhibition do not necessarily correlate with doses required for efficacy in vivo. A biological assay, such as the reverse passive Arthus reaction, is perhaps a better method of determining effective dosages.

Conclusions. CLB54 administered at doses >10 mg/kg reduces infarct size in a baboon model of 90 min of ischemia followed by 4 h of reperfusion. Although the mechanism of benefit appears to be related to reduced sequestration of neutrophils in the myocardium, other potential contributing mechanisms cannot be excluded. Although interference with the normal process of inflammation appears necessary to minimize reperfusion injury, deleterious effects on infarct healing, leading to aneurysm formation or myocardial rupture, and increased susceptibility to infection may result. Further long-term studies in animal models will be required to define both the duration of benefit and the potentially harmful consequences of such treatment.

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